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(57) Abstract

A polypeptide immunogen is provided which polypeptide stimulates the production of neutralizing antibody to rotavirus strains. The polypeptide comprises an epitope of a conserved region of rhesus rotavirus (RRV) VP3 or VP7 protein. Usually, the polypeptide comprises at least 10 amino acides having substantially the same amino acid sequence as consecutive amino acids within residues 75 to 200 or 370 to 490 of VP3 or residues 75 to 250 of VP7. DNA fragments and expression constructs encoding the polypeptides are also provided, together with vaccines and methods of protecting a susceptible host animal from rotavirus infection.

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CONSERVED ROTAVIRUS GENE SEGMENTS AND USE IN IMMUNIZATION AND NEUTRALIZATION

INTRODUCTION

Technical Field

The present invention relates to viral vaccines and in particular to rotavirus vaccines.

Background Of The Invention

Rotaviruses have been established as an important cause of severe gastroenteritis in humans as well as in other mammalian and avian species. Although rotaviral disease is most common in children under two years of age, it also affects the elderly and the immunocompromised. The public health and economic effects of rotavirus disease have made the need for a vaccine apparent, especially in less developed countries where insufficient medical care often results in infant mortality (Shaw et al., Virology (1986) 155:434-451).

The rhesus strain of simian rotavirus (RRV) is serotypically identical to serotype 3 human rotavirus strains (Hoshino et al., J. Infect. Dis. (1984) 149:694-701). Although the strain grows well in culture and appears to be attenuated in humans (Kapikian et al., In: "Modern Approaches to Vaccines Molecular and Chemical Basis of Resistance to Viral, Bacterial, or Parasitic Diseases" (1985) 357-367), it is preferable to use synthetic viral proteins or immunogenic portions thereof rather than using intact, attenuated virus to minimize potential adverse side effects of a vaccine. However, the antigenic characteristics of the surface proteins are not sufficiently well defined at

the present time to allow the production of such a synthetic vaccine.

Viral protein 3 (VP3), a surface protein of RRV, has been identified as containing the rotaviral hemagglutinin (Kalica et al., Virology (1983) 125:194-205). VP3 is also associated with restriction of virulence of certain rotavirus strains in mice (Offit et al., J. Virol. (1986) 57:376-378) and humans (Flores et al., J. Virol. (1986) 60:972-979). In vitro cleavage of VP3 by trypsin produces two new surface proteins of approximately 60 and 28 kD, VP5 and VP8, respectively, which result in enhanced viral infectivity (Estes et al., J. Virol. (1983) 39:879-888). Antibody to VP3 has been shown to inhibit viral hemagglutination in vitro (Greenberg et al., Infect. Immun. (1983) 39:91-99), to neutralize rotaviruses in vitro and to passively protect mice against heterologuos rotavirus challenge in vivo (Offit et al., J. Virol. (1986) 58:700-703). In addition, VP3 induces protective immunity in animals (Offit et al., J. Virol. (1986) 60:491-496).

VP7 is a 34 to 38 kD glycoprotein. Antibodies to VP7 neutralize the virus and specify the viral serotype (Bastardo et al., Infect. Immun. (1981) 34:64-647; Kalica et al., Virology (1981) 112:385-390; Dyall-Smith et al., In: "Infectious Diarrhea in the Young - Strategies for Control in Humans and Animals" (1985), Elsevier Science, 215-220; Greenberg et al., J. Virol. (1983) 64:313-324; Greenberg et al., J. Virol. (1983) 47:267-275; Matsuno et al., Infect. Immun. (1983) 39:879-888).

Definitive localization of serotype-specific and cross-reactive neutralizing domains awaits amino acid sequence analysis of the protein and its variants.

Relevant Literature

Offit et al., J. Virol. (1986) 58:700-703 describe experiments where suckling mice given neutralizing monoclonal antibodies orally 30 min. before oral challenge with rotavirus were protected by the monoclonal antibodies. The authors concluded that higher plaque reduction neutralization titers correlated with in vivo protection. Offit et al., J. Virol (1986) 60:491-496 used genetic studies of reassortant rotaviruses to determine which viral genes coded for proteins which induced a protective immune response in vivo. The authors concluded that both VP3 and VP7 appear to act independently in stimulating a protective immune response against rotavirus challenge for a passive or active immune response in mice challenged with homologous or heterologous host rotaviruses. The authors report that this differs from large scale human clinical trials where the ability to demonstrate heterotypic protection against challenge in a passive model was not demonstrable. Shaw et al., Virology (1986) 155:434-451 report antigenic mapping with a plurality of monoclonal antibodies directed to surface proteins of rhesus rotavirus.

SUMMARY OF THE INVENTION

A polypeptide immunogen is provided which polypeptide stimulates the production of neutralizing antibody to rotavirus strains. The polypeptide comprises an epitope of a conserved region of rhesus rotavirus (RRV) VP3 or VP7 protein. Usually the polypeptide comprises at least 10 amino acids having substantially the same amino acid sequence as consecutive amino acids within residues 75 to 200 or 370 to 490 of VP3 or residues 75 to 250 of VP7. DNA fragments and expression constructs encoding the polypeptides are also provided, together with vaccines and methods of protecting a susceptible host animal from rotavirus infection.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention provides a polypeptide composition which stimulates the production of antibodies that neutralize a broad spectrum of rotavirus strains. The polypeptides comprise epitopes within conserved regions of rhesus rotavirus (RRV) VP3 protein or VP7 protein. DNA fragments and expression constructs encoding the polypeptides are also provided, together with vaccines and methods of protecting a susceptible host animal from rotavirus infection.

The primary nucleotide sequence of RRV gene 4 and gene 9, and deduced amino acid sequence of the encoded proteins have now been determined. Those sequences are illustrated in Tables 2 and 5, respectively. (Throughout the specification and in the claims, references to the RRV gene 4 sequence or the RRV gene 9 sequence, or the sequences of the encoded proteins mean the sequences which are described in Tables 2 and 5.) The sequences were used to map the antigenic characteristics of the VP3 and VP7 surface proteins.

Two large hydrophobic regions within amino acids 370-490, amino acid residues 380-416 and 451-480 are conserved sequences within region 6 of VP5 which induce antibodies that neutralize a wide variety of serotypically distinct rotavirus strains. Within residues 75-200 of VP8 are a number of sequences which induce antibodies of limited strain specificity. That is, the antibody is more serotype-specific than that induced by the region 6 sequences. Additionally, amino acids 75-250 are conserved regions in VP7 which induce neutralizing antibodies.

In particular, it has been found that the alteration of a single amino acid at particular residues in region 6 of VP5 was sufficient to let a viral variant escape neutralization with a neutralizing mono-

clonal antibody. The particular altered amino acid therefore comprises a portion of the epitope to which neutralizing antibody binds. Those amino acids will be referred to as neutralizing-region amino acids. Residues 87-89, 100, 114, 135, 148, 150, 173 and 188 have now been shown to be neutralizing-region amino acids of the serotype-specific region of VP8. Alterations at residues 388 and 393 in the conserved region of VP5 and at residues 94, 96, 97, 99, 211 and 212 of VP7 also allowed variants to escape neutralization.

A polypeptide of this invention comprises at least 10 amino acids having the same amino acid sequence as consecutive amino acids within residues 75-200 or 370-490 of VP3 or 75-250 of VP7 of rhesus rotavirus and sequences immunologically cross-reactive therewith. By immunologically cross-reactive therewith is meant that the cross-reactive sequences are bound by antibodies which bind RRV and that antibodies which bind the cross-reactive sequence bind to RRV. A polypeptide of this invention usually has the same amino acid sequence as at least about 15 consecutive amino acids located within residues 75-200, 380-416 or 451-480 of RRV VP3 or 75-125 or 200-230 of RRV VP7 and not more than 3 single site lesions, usually no more than one single site lesion. By a single site lesion is meant a lesion involving one amino acid.

Usually, the polypeptides include at least one neutralizing-region amino acid, e.g. residues 87-89, 100, 114, 135, 148, 150, 173, 388 and 393 of VP3 and 94, 96, 97, 99, 211, and 212 of VP7. More usually, the polypeptides will include 5, most usually 10, amino acids on each side of the neutralizing-region amino acid. Desirably, a vaccine will not let minor variants escape neutralization. Thus a vaccine polypeptide will usually not differ from the wild type sequence at neutralizing region amino acids.

Immunologically cross-reactive polypeptides may have a sequence identical to a portion of the RRV sequence. In some situations the sequence may contain one or more lesions. That is, one or more amino acids can be either substituted for an amino acid in the sequence or inserted into or deleted from the sequence. Usually the alterations comprise not more than about 10% of the sequence. Insertions, deletions or substitutions will usually involve no more than about 5 amino acids, more usually less than 3 amino acids, most usually 1 to 2 amino acids. Lesions will generally be conservative substitutions. To determine whether a polypeptide is cross-reactive with a VP3 or VP7 sequence, the polypeptide can be tested for binding with anti-VP3 or anti-VP7 antibodies, preferably neutralizing antibodies. Alternatively, peptide-induced antibodies can be tested for binding to, preferably neutralization of, RRV. By neutralization, it is meant that when the antibody is bound to the virus, the virus is no longer infectious. A neutralization assay is described in Shaw et al., Virology (1986) 155:434-451 and the references cited therein. Other assays to determine neutralization are well known.

The present invention provides substantially pure preparations of VP3 and VP7 or immunologically active fragments thereof. By substantially pure is meant that the preparation is free from other rhesus rotavirus proteins. By immunologically active is meant the fragment is antigenic. The fragments comprise not more than about 90%, usually not more than about 75%, most usually not more than about 50% of the protein sequence. Conveniently, the fragment has fewer than about 100 amino acids, generally ranging from about 15 amino acids to about 35 to 50 amino acids.

The subject polypeptides may be prepared in variety of ways. Polypeptides may be synthesized in

accordance with conventional synthetic techniques, particularly automated synthesizers, or may be prepared by recombinant techniques where a DNA sequence encoding the gene or a portion thereof is inserted into an expression vector for expression in a host cell. Particularly where the polypeptide will be joined to one or more other peptide sequences, the recombinant techniques may be employed with advantage.

Various expression vectors are commercially available or have been described in the literature, or alternatively may be prepared. The expression construct comprises a DNA sequence encoding VP3 or VP7 or fragments or combinations thereof under the transcriptional control of the native or other than the native promoter. The expression cassette comprises in the direction of transcription, a transcriptional and translational initiation region, the open reading frame with initiation and stop codons and a translational and transcriptional termination region. The expression vector may be part of a vector capable of stable extrachromosomal maintenance in an appropriate cellular host or may have DNA homologous with the chromosome of the host for insertion. Alternatively, the expression vector may be bordered by sequences which allow for insertion into a host, such as transposon sequences, lysogenic viral sequences, or the like. Desirably, markers are provided with the expression vector which allows for a selection of host cells containing the expression vector. The marker may be on the same or a different DNA molecule, desirably the same DNA molecule.

The DNA may be introduced into the host by any convenient means, including fusion, conjugation, transfection, transduction, electroporation, injection, or other convenient means. Selection of host cells containing the expression vector may then be determined by means of the marker. Convenient markers include

resistance to a cytotoxic agent, complementation of an auxotrophic host to prototrophy, production of a detectable product, etc.

The transformed cells may be screened and positive clones expanded and used for expression of the polypeptide. Expression vectors can be provided which allow for a secretion of the polypeptide by joining the polypeptide in reading frame with the signal sequence and processing signal which allows for secretion of the polypeptide and cleavage of the signal sequence at the processing site from the peptide. These techniques may be found in a wide number of patents, patent applications, and scientific articles. See for example, U.S. Patent Numbers 4,599,308, 4,601,980, 4,612,287 and 4,615,974.

Additionally, the polypeptides may be expressed as fusion proteins. The open reading frame encoding the second polypeptide will be joined in reading frame with the gene 4 or gene 9 sequence to provide a fusion protein. The sequence encoding the second polypeptide may provide a promoter or encode a signal sequence. The second polypeptide may provide a convenient marker for identifying clones expressing the protein and, desirably, may include one or more immunodominant sequences to provide enhanced immunogenicity to the RRV peptide. Usually, the second polypeptide will be a heterologous protein, i.e. other than an RRV protein, or fragment thereof having at least about 15 amino acids.

A wide variety of hosts may be employed for expression of the polypeptides, both prokaryotic and eukaryotic. Useful hosts include bacteria, such as E. coli, yeast, filamentous fungus, immortalized mammalian cells, such as various mouse lines, monkey lines, human lines or the like. For the most part, the mammalian lines will be immortalized by transformation to a neoplastic state, where the cells may be isolated from the

neoplastic host, or wild-type cells may be transformed with oncogenes, tumor-causing viruses, or the like. Depending on the presence of the secretory signal sequence, the peptide may be isolated from the supernatant in which the expression host is grown or from a lysate of the expression host. The peptide may then be isolated by conventional techniques employing HPLC, electrophoresis, gradient centrifugation, affinity chromatography, etc. to provide a substantially pure product, particularly free of cell component contaminants.

One or more of the polypeptides can be used as a vaccine to protect a host animal or to induce neutralizing antibodies which are protective on passive immunization. The vaccines of the present invention comprise a physiologically suitable diluent containing an effective amount of a polypeptide composition having at least one polypeptide of the present invention. Conveniently, the vaccines contain a plurality of the polypeptides, usually a mixture of a peptide from residues 370-490 together with one or more peptides from residues 75-200 and may include peptides from VP7.

Polypeptides are having less than about 100 amino acids are generally haptenic and must be made immunogenic for use as a vaccine. The peptides can be joined together or bound to immunogenic RRV protein fragments or a carrier to produce an immunogen. Suitable carriers for immunization vary with the host animal and are well known. Additionally the peptide or peptide-carrier conjugate can be used with an adjuvant to enhance the immune response.

The peptide vaccines may be formulated in any convenient physiologically acceptable medium for administration to a host. These media include milk, formula, water, saline, phosphate buffered saline, oil emulsions, etc. These formulations are well known in the literature. Administration will usually be oral

but may also be by injection, for example, intravascular, peritoneally, subcutaneously, subtopically, intradermal patches, etc.

The amount of the subject compositions will vary depending upon the manner of administration, the host, the frequency of repetitive treatment, if any, and the like. For the most part, with each composition, the amount used will not differ from amounts used for other peptide vaccines using the same carrier and diluent. The peptides will generally range from about 0.1 to 20 µg/kg of host, where concentrations will generally range from about 0.1 µg/ml to 10 mg/ml. Other additives may be included in the formulations, such as stabilizers, antibiotics, excipients, adjuvants, precipitates for adsorption, slow release additives, etc.

The vaccines can be used to protect a susceptible host animal from rotavirus infection by administering an effective amount of the vaccine to the host animal. The administration may be repeated on one or more occasions, usually one or two times at least about two to six weeks after the initial administration and again at one to ten year intervals. The vaccine will usually be administered orally to elicit gastrointestinal tract IgA. The peptides will be administered in a specially formulated enteric composition such as milk, formula and other solutions buffered to reduce the effect of stomach acid on the peptides. However, intramuscular administration is also contemplated. Additionally, antibodies induced by the vaccines can be passively administered to a host animal, usually an infected host.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Gene 4

Materials and Methods

Cells and Viruses

MA104 cells are a continuous line of rhesus monkey kidney cells and were grown in medium 199 (Irvine Scientific) as previously described in Shaw et al., Microbiol. (1985) 22:286-291. Rhesus rotavirus was activated in 5 µg/ml trypsin (Sigma type IX) for 1 hour at 37°C, adsorbed onto MA104 monolayers for 1 hour and grown for 2-3 days in the presence of trypsin (0.5 µg/ml). Virus was harvested, extracted and purified on CsCl gradients as previously described in Shaw et al., supra.

Production of VP3 Rotavirus Variants

The production, characterization and selection of variants with VP3 specific neutralizing monoclonal antibodies (mAbs) 5C4, 1A9, 7A12, and 2G4 was previously described in Shaw et al., Virology (1986) 155:434-451. Additional VP3 N-mAbs A1, A15, M11, 5D9, M14, M7, and M2 were chosen to select new variants because they were not serologically related to the previous library of escape mutants (see Table 1).

TABLE 1

GENE 4 NUCLEOTIDE AND AMINO ACID CHANGES IN VARIANTS SELECTED BY N-MABS

RRV VARIANT	NUCLEOTIDE CHANGE bp #	AMINO ACID CHANGE (AA#)	REGION	SELECTING MAB NEUTRALIZATION SPECIFICITY*
M11	A → G bp 268	Thr → Ala (87)	1	RRV
A1	C → A bp 272	Thr → Lys (88)	1	RRV, NCDV
A15	G → C bp 274	Ala → Pro (89)	1	RRV
1A9	G → A bp 307	Asp → Asn (100)	2	RRV
5D9	C → T bp 350	Ser → Phe (114)	3	RRV
M14	A → G bp 452	Gln → Arg (148)	4	RRV, NCDV
5C4	G → A bp 458	Gly → Glu (150)	4	RRV
7A12	A → T bp 572	Tyr → Phe (188)	5	RRV
M2	C → A bp 1172	Ala → Glu (388)	6	RRV, SA11, UK, Wa, Got
2G4	A → C bp 1187	Gln → Pro (393)	6	RRV, SA11, OSU, UK
M7	A → C bp 1187	Gln → Pro (393)	6	RRV, SA11, UK
GAAD†	G → A bp 307	Asp → Asn (100)		
	A → G bp 413	Gln → Arg (135)		
	A → G bp 527	Lys → Arg (173)		
	A → C bp 1187	Gln → Pro (393)		

* N-mAbs that selected variants were tested in plaque reduction neutralization tests (focus reduction neutralization for DS1) (Shaw et al., Virology (1986) 155:434-451). Neutralizing mAbs were titered against RRV, SA11, NCDV, OSU, UK DS1, WA, and Gottfried rotavirus strains. Viral neutralization is designated if the titer of the mAb against the indicated viruses is no less than 1/8 of the titer against RRV.

† GAAD was constructed as a quadruple RRV mutant made sequentially to N-mAbs 2G4, 1A9, 7A12, and 5D9.

The selection was carried out as before in Shaw et al., supra. A sequential series of double, triple and quadruple variants were also selected. Variant 2G4 was passaged 4 times in the presence of N-mAb 1A9 and then 2G4/1A9 variants were isolated by plaque purification as described in Shaw et al., supra. Subsequently, the same procedure was employed with N-mAb 7A12 and then 5D9. Each variant was resistant to each of the selecting N-mAbs. Triple plaque purified variants were passed two to three times in order to produce high titer stocks for virus purification.

In vitro RNA Transcription

Density gradient purified double shelled particles were converted to polymerase active single shelled cores by incubation for 30 minutes at 40°C in 10 mM Tris-HCl pH 8.0, 10 mM EDTA. The endogenous RNA polymerase activity of the activated cores was then used to synthesize plus stranded RNA's in vitro as described by Flores et al., Virology (1982) 121:288-295. Following phenol extraction, ssRNA was separated from double stranded RNA by 2 M LiCl precipitation and subsequently ethanol precipitated.

RNA Sequencing

Total ssRNA was initially fractionated on a 1% low melting agarose gel and the gene 4 segment was isolated. Approximately 20 adenine residues were added to the 3' end of gene 4 using poly A polymerase (Bethesda Research Labs, BRL) as described by Sippel, Eur. J. Biochem. (1973) 37:31. With the knowledge of the conserved 3' terminal sequence of rotavirus genes (Both et al., Nucleic Acids Res. (1982) 10:7075-7088), the oligonucleotide 5' TTTTTTTTTTTTTGG 3' was used to prime the initial Sanger dideoxynucleotide sequencing reactions (Sanger et al., Proc. Natl. Acad. Sci. USA (1977) 74:5463-5467).

Primers (100 ng) were mixed with approximately 5 µg of total plus stranded rotavirus RNA from the polymerase reaction, boiled for 1 minute and quick chilled on ice. Sequencing reactions containing 1.0 unit/µl RNasin (Promega Biotec) were carried out in reaction mixtures as described by Emini et al., Nature (London) (1983) 304:699-703, or Zagursky et al., Gene Anal. Tech. (1985) 2:5 in the presence of 0.5 unit/µl of AMV reverse transcriptase (Seikagaku America). Reactions were terminated in a 95% formamide dye mixture, heated at 90°C for 3 minutes and run on 5% polyacrylamide sequencing gels. The sequence was extended across gene 4 by making negative strand primers, 18 nucleotides in length, on an Applied Biosystems DNA synthesizer at intervals of 200 to 250 nucleotides.

In order to verify a sequence in regions of high secondary structure, additional primers of positive or negative polarity were used to deduce the entire nucleotide sequence of gene 4. Primers of positive polarity were annealed to double stranded (ds) RNA templates denatured by the method of Bassel-Dubey et al., J. Virol. (1986) 60:64-67 and sequenced as above. Sequence in the VP8 portion of gene 4 was also determined by direct plasmid sequencing (Zagursky et al., supra) of 2 overlapping cDNA clones.

The 5' terminal sequences were verified by direct enzymatic RNA sequencing (Donis-Keller, Nucleic Acids Res. (1977) 4:2527). Briefly, the 5 ends of dsRNA were labeled with cytidine 3', 5'-[5'-^{32}P] bisphosphate in the presence of T4 RNA ligase. An excess of cold ssRNA was annealed to the dsRNAs as described by Skehel et al., Virology (1969) 39:822 and the ds and ss RNAs were separated by 2M LiCl precipitation of the ssRNA. Enzymatic RNA sequencing was carried out using base specific enzymes, RNase T1, RNase U2, RNase Phy M, RNase B. cereus and RNase CL3 (BRL), and separated on 20% polyacrylamide gels.

Results

Sequence of RRV Gene 4, VP3

The complete nucleotide sequence of the rhesus rotavirus gene 4 is presented in Table 2.

TABLE 2

SEQUENCE OF RRV GENE 4, VP3

		1	GGC	TAT	AAA	ATG	GCT	TCG	CTC	ATT	TAT	AGA	CAA
	RRV	1				M	A	S	L	I	Y	R	Q
	SA11							A					
			TTG	CTT	ACA	AAT	TCA	TAT	ACC	GTT	GAC	CTA	TCT
			L	L	T	N	S	Y	T	V	D	L	S
											E		
			GAT	GAA	ATA	CAA	GAA	ATT	GGA	TCT	ACT	AAG	ACG
			D	E	I	Q	E	I	G	S	T	K	T
			CAA	AAT	GTC	ACT	ATT	AAT	CTA				
			Q	N	V	T	I	N	L				
							V		P				
		121	GGA	CCC	TTC	GCG	CAA	ACA	GGT	TAT	GCT	CCA	GTT
	RRV	38	G	P	F	A	Q	T	G	Y	A	P	V
	SA11												
			AAC	TGG	GGT	CCT	GGT	GAA	ACT	AAT	GAT	TCT	ACT
			N	W	G	P	G	E	T	N	D	S	T
			ACT	GTA	GAA	CCG	GTA	CTT	GAT	GGT	CCT	TAT	CAA
			T	V	E	P	V	L	D	G	P	Y	Q
			CCA	ACT	TCG	TTC	AAT	CCA	CCA				
			P	T	S	F	N	P	P				
					T								
		241	GTA	GAT	TAT	TGG	ATG	CTA	TTA	GCA	CCT	ACA	GCA
	RRV	78	V	D	Y	W	M	L	L	A	P	T	A
	SA11			S									N
			GCT	GGA	GTA	GTA	GTA	GAA	GGA	ACT	AAT	AAT	ACA
			A	G	V	V	V	E	G	T	N	N	T
			GAC	CGA	TGG	CTA	GCT	ACA	ATT	TTA	GTT	GAG	CCT
			D	R	W	L	A	T	I	L	V	E	P
			N								I		
			AAC	GTA	ACA	TCA	GAA	ACC	AGA				
			N	V	T	S	E	T	R				
					Q	Q	V	E					

TABLE 2 (continued)

		361	AGT	TAT	ACG	CTA	TTT	GGA	ACG	CAA	GAG	CAA	ATT
	RRV	118	S	Y	T	L	F	G	T	Q	E	Q	I
	SA11		T						Q		V		V
			ACA	ATA	GCT	TAT	GCT	TCC	CAA	ACA	CAA	TGG	AAA
			T	I	A	Y	A	S	Q	T	Q	W	K
				V	S	N	D				K		
			TTT	ATT	GAT	GTC	GTT	AAA	ACT	ACA	CAA	AAT	GGA
			F	I	D	V	V	K	T	T	Q	N	G
				V		L	S		Q			D	
			AGC	TAT	TCA	CAA	TAC	GGA	CCA				
			S	Y	S	Q	Y	G	P				
			N				H		S				
		481	TTA	CAA	TCT	ACT	CCA	AAA	CTC	TAT	GCC	GTG	ATG
	RRV	158	L	Q	S	T	P	K	L	Y	A	V	M
	SA11			L							G		
			AAA	CAT	AAT	GGT	AAA	ATT	TAT	ACA	TAT	AAT	GGA
			K	H	N	G	K	I	Y	T	Y	N	G
					G								
			GAA	ACT	CCG	AAT	GTG	ACC	ACT	AAG	TAC	TAC	TCA
			E	T	P	N	V	T	T	K	Y	Y	S
							A	N		G			
			ACT	ACA	AAT	TAT	GAT	TCA	GTA				
			T	T	N	Y	D	S	V				
						F		T					
		601	AAC	ATG	ACA	GCA	TTT	TGT	GAC	TTT	TAT	ATT	ATA
	RRV	198	N	M	T	A	F	C	D	F	Y	I	I
	SA11						Y						
			CCT	AGA	GAA	GAA	GAA	TCA	ACA	TGT	ACC	GAG	TAC
			P	R	E	E	E	S	T	C	T	E	Y
				L	A	Q		A	K				
			ATT	AAT	AAC	GGG	TTA	CCT	CCG	ATT	CAG	AAT	ACA
			I	N	N	G	L	P	P	I	Q	N	T
			CGA	AAC	ATT	GTT	CCA	TTG	GCG				
			R	N	I	V	P	L	A				
								V	S				

TABLE 2 (continued)

		721	CTT	TCA	GCT	AGA	AAT	ATA	ATA	TCA	CAT	AGA	GCT
	RRV	238	L	S	A	R	N	I	I	S	H	R	A
	SA11		I	V	S				V	S	T		
			CAA	GCG	AAT	GAA	GAT	ATC	GTT	GTG	TCA	AAG	ACA
			Q	A	N	E	D	I	V	V	S	K	T
				P		Q							
			TCA	CTT	TGG	AAA	GAG	ATG	CAA	TAC	AAT	AGA	GAC
			S	L	W	K	E	M	Q	Y	N	R	D
			ATC	ACA	ATT	CGA	TTT	AAA	TTC				
			I	T	I	R	F	K	F				
				V									
		841	GCA	AGT	TCA	ATT	GTT	AAA	TCC	GGT	GGG	CTA	GGT
	RRV	278	A	S	S	I	V	K	S	G	G	L	G
	SA11			N			I						
			TAT	AAA	TGG	TCA	GAG	ATT	TCA	TTT	AAA	CCA	GCA
			Y	K	W	S	E	I	S	F	K	P	A
								V					
			AAC	TAT	CAA	TAT	ACG	TAT	ACA	CGA	GAT	GGA	GAG
			N	Y	Q	Y	T	Y	T	R	D	G	E
			F										
			GAT	GTT	ACA	GCT	CAC	ACG	ACG				
			D	V	T	A	H	T	T				
			E										
		961	TGC	TCA	GTA	AAC	GGA	ATG	AAC	GAT	TTT	AAT	TTC
	RRV	318	C	S	V	N	G	M	N	D	F	N	F
	SA11							V					Y
			AAT	GGG	GGA	TCG	TTA	CCA	ACG	GAT	TTT	ATA	ATA
			N	G	G	S	L	P	T	D	F	I	I
												V	
			TCA	AGA	TAT	GAA	GTA	ATT	AAA	GAG	AAT	TCT	TAT
			S	R	Y	E	V	I	K	E	N	S	Y
				K									F
			GTT	TAT	GTT	GAT	TAC	TGG	GAT				
			V	Y	V	D	Y	W	D				
					I								

TABLE 2 (continued)

RRV SA11	1081	GAT TCA CAA GCC TTC AGG AAC ATG GTT TAT GTA	
	358	D S Q A F R N M V Y V	
		AGG TCA TTA GCT GCT AAT TTA AAC TCT GTT ATA	
		R S L A A N L N S V I	
		TGT ACT GGG GGT GAT TAT AGC TTT GCA TTA CCG	
		C T G G D Y S F A L P	
		GTT GGT CAA TGG CCA GTA ATG	
		V G Q W P V M	
		N Y	
RRV SA11	1201	ACT GGC GGA GCA GTG TCA TTG CAT TCA GCT GGT	
	398	T G G A V S L H S A G	
		GTT ACG TTA TCC ACA CAG TTC ACA GAT TTT GTA	
		V T L S T Q F T D F V	
		TCA TTT AAT TCT TTA AGG TTC AGG TTT AGA CTA	
		S F N S L R F R F R L	
		ACT GTT GAA GAG CCA TCA TTC	
		T V E E P S F	
		S P	
RRV SA11	1321	TCG ATC ACC AGA ACT AGA GTT GGT GGA TTG TAT	
	438	S I T R T R V G G L Y	
		L S W	
		GGG TTA CCT GCA GCT TAC CCC AAT AAT GGA AAA	
		G L P A A Y P N N G K	
		K S Q	
		GAA TAT TAT GAA GTG GCT GGC AGA CTC TCA CTA	
		E Y Y E V A G R L S L	
		I F	
		ATA TCA TTG GTA CCA TCT AAT	
		I S L V P S N	
		L	

TABLE 2 (continued)

		1441	GAC	GAT	TAC	CAG	ACA	CCA	ATA	ACT	AAT	TCA	GTT
	RRV	478	D	D	Y	Q	T	P	I	T	N	S	V
	SA11										M		
			ACA	GTC	AGA	CAA	GAT	TTA	GAA	CGA	CAG	TTG	GGT
			T	V	R	Q	D	L	E	R	Q	L	G
			GAA	CTT	AGA	GAA	GAA	TTC	AAC	GCT	CTC	TCA	CAA
			E	L	R	E	E	F	N	A	L	S	Q
						D				N			
			GAG	ATA	GCC	ATG	TCG	CAG	CTT				
			E	I	A	M	S	Q	L				
			Q										
		1561	ATT	TAT	TTG	GCA	TTA	CTT	CCA	TTG	GAT	ATG	TTT
	RRV	518	I	Y	L	A	L	L	P	L	D	M	F
	SA11			D									
			TCG	ATG	TTT	TCT	GGT	ATT	AAG	AGC	ACC	ATA	GAT
			S	M	F	S	G	I	K	S	T	I	D
			GCA	GCT	AAA	TCA	ATG	GCT	ACT	AGT	GTA	ATG	AAG
			A	A	K	S	M	A	T	S	V	M	K
										N			
			AAA	TTT	AAG	AAA	TCA	GGT	TTA				
			K	F	K	K	S	G	L				
			R					S					
		1681	GCT	AAC	TCT	GTA	TCT	ACA	TTA	ACA	GAC	TCA	CTG
	RRV	558	A	N	S	V	S	T	L	T	D	S	L
	SA11												
			TCC	GAC	GCA	GCT	TCT	TCA	ATT	TCA	AGA	GGA	GCA
			S	D	A	A	S	S	I	S	R	G	A
												S	
			TCT	ATT	CGT	TCA	GTT	GGA	TCA	TCA	GCA	TCA	GCA
			S	I	R	S	V	G	S	S	A	S	A
				V				S		T			
			TGG	ACG	GAT	GTC	TCA	ACA	CAA				
			W	T	D	V	S	T	Q				
					E			N	I				

TABLE 2 (continued)

		1801	ATC	ACT	GAT	GTT	TCT	TCA	TCT	GTC	AGT	TCG	ATC
	RRV	598	I	T	D	V	S	S	S	V	S	S	I
	SA11		A	S		I	N	V	T	T			
			TCG	ACA	CAG	ACT	TCA	ACT	ATT	AGT	AGA	CGG	CTA
			S	T	Q	T	S	T	I	S	R	R	L
			CGA	CTA	AAA	GAA	ATG	GCT	ACG	CAA	ACA	GAA	GGG
			R	L	K	E	M	A	T	Q	T	E	G
												D	
			ATG	AAT	TTC	GAT	GAT	ATA	TCT				
			M	N	F	D	D	I	S				
		1921	GCT	GCA	GTA	TTG	AAG	ACT	AAA	ATT	GAT	CGA	TCC
	RRV	638	A	A	V	L	K	T	K	I	D	R	S
	SA11											K	
			ACT	CAA	ATA	TCT	CCA	AAC	ACA	TTA	CCA	GAT	ATA
			T	Q	I	S	P	N	T	L	P	D	I
					L	N	T					E	
			GTC	ACT	GAA	GCT	TCA	GAG	AAG	TTT	ATT	CCT	AAT
			V	T	E	A	S	E	K	F	I	P	N
			AGA	GCG	TAC	AGA	GTA	ATT	AAT				
			R	A	Y	R	V	I	N				
									K				
		2041	AAT	GAT	GAA	GTC	TTT	GAA	GCG	GGA	ACA	GAT	GGA
	RRV	678	N	D	E	V	F	E	A	G	T	D	G
	SA11		D				L			S	I		
			AGA	TAT	TTT	GCG	TAT	CGT	GTT	GAA	ACG	TTC	GAT
			R	Y	F	A	Y	R	V	E	T	F	D
			K				D	K					E
			GAA	ATT	CCA	TTT	GAT	GTG	CAA	AAG	TTT	GCA	GAT
			E	I	P	F	D	V	Q	K	F	A	D
			CTA	GTA	ACT	GAC	TCT	CCG	GTC				
			L	V	T	D	S	P	V				

TABLE 2 (continued)

		2161	ATC	TCA	GCC	ATT	ATA	GAC	TTT	AAG	ACA	CTC	AAG
	RRV SA11	718	I	S	A	I	I	D	F	K	T	L	K
			AAT	CTA	AAC	GAC	AAT	TAT	GGT	ATT	AGT	AGG	CAA
			N	L	N	D	N	Y	G	I	S	R	Q
			CAA	GCA	TTT	AAT	CTG	CTA	AGA	TCC	GAT	CCA	AGA
			Q	A	F L	N	L	L	R	S	D	P	R
			GTA	TTA	CGT	GAA	TTT	ATC	AAT				
			V A	L	R	E	F	I	N				
		2281	CAA	GAC	AAT	CCA	ATA	ATT	CGT	AAC	AGA	ATT	GAA
	RRV SA11	758	Q	D	N	P	I	I	R	N	R	I	E
			CAG	TTA	ATA	ATG	CAG	TGT	AGA	CTG	TAA	GCA	ATT
			Q S	L	I	M	Q	C	R	L			
			TCT	AGA	GGA	TGT	GAC	C					

The RRV gene 4 RNA sequence is presented in the DNA form along with the encoded VP3 amino acid sequence. Changes in the SA11 VP3 amino acid sequence (Lopez et al., Virology (1985) 144:11-19 and Lopez et al., Nucleic Acids Res. (1987) 15:4691) appear below that of RRV. Cysteines are boxed and long hydrophobic amino acid (AA) regions are underlined. Codons are overlined in which single nucleotide substitutions were detected in N-mAb selected variants. Potential trypsin cleavage sites that divide VP3 into VP8 and VP5 are indicated by arrows.

Gene 4 contained one long open reading frame which originated 10 nucleotides from the 5' end and terminated with a single stop codon 22 bases from the 3' end. The ATG start codon conformed to Kozak's rules (Kozak, Nucleic Acids Res. (1981) 9:5233) for strong translation initiation (AXX ATGG). There is no potential poly A addition signal following the termination codon. The encoded protein, VP3, was comprised of 776 amino acids (AA) with a calculated molecular weight of 86.5 kD. The preferred trypsin cleavage site identified by Lopez et al., Virology (1985) 144:11-19 was conserved, yielding a VP8 of 247 amino acids (27 kD) and VP5 of 529 AA (60 kD). Two other potential trypsin cleavage sites preceded the preferred site.

The RRV nucleotide and amino acid sequences were compared with the sequence of simian rotavirus, SA11 (Lopez et al., Virology (1985) 144:11-19 and Lopez et al., Nucleic Acids Res. (1987) 15:4691) (Table 2) and with the human strain, RV-5 (Kantharidis et al., Arch. Virol. (1987) 93:111-121). At the nucleotide level, gene 4 of RRV and SA11 were 74% homologous while RRV and RV-5 were 67% related. In addition, conservation of nucleotide sequence was maintained for 33 bases at the 5' terminus of RRV, SA11 and RV-5 (2 bases differ in SA11), but only 11 bases were identical at their 3' termini.

At the amino acid level, RRV was more closely related to SA11 than to RV-5. There was 84% total amino acid homology between RRV and SA11 with 78% AA homology in VP8 and 88% AA homology in VP5. RRV and RV-5 shared 72% homology at the amino acid level; 58% in VP8 and 78% in VP5. Only 5 cysteines existed in the RRV VP3. These were all conserved with those in the SA11 VP3 sequence. RV-5 lacked the cysteine at AA 203 but contained the 4 other cysteine residues in VP3. Unlike SA11 and RV5, there was no potential chymotrypsin cleavage site located between the RRV VP3 trypsin cleavage sites. The area between the cleavage sites was highly divergent in all three strains. All three viral proteins contain long regions of identical residues at AA's 4-14, 56-72, 222-235, 257-271, 346-359, 361-378, 407-420, 456-468, 521-540 and 711-736. VP5 also contained one conserved hydrophobic region of 20 AA (385 to 404) which could serve a membrane spanning role (Table 2).

Selection of Variants Resistant to Neutralizing mAbs

Monoclonal antibodies directed at VP3 were originally selected for their ability to inhibit hemagglutination, neutralize RRV and immunoprecipitate VP3 from a cell lysate (Shaw et al., Virology (1986) 155:434-451). Variants resistant to individual N-mAbs were selected from the parental strain, RRV, and named for the selecting N-mAb (Table 3). Representative variants selected by serologically distinct groups of N-mAbs were chosen for sequence analysis (Table 1).

TABLE 3

RESISTANCE OF ROTAVIRUS VARIANTS TO NEUTRALIZING MONOCLONAL ANTIBODIES

		RESISTANCE OF VARIANTS TO HEMAGGLUTINATION INHIBITION AND NEUTRALIZATION*											
		VARIANTS TESTED†											
REGION	N-MAB	M11	A1	A15	1A9	5D9	M14	5C4	7A12	M2	2G4	M7	GAAD
1	M11	R	R										
1	A1		R	R									
1	A15	R	R	R									
2	1A9			R	R								R
3	5D9					R							R
4	M14						R	R					
4	5C4				R			R					R
5	7A12								R				R
6	M2									R			
6	2G4										R	R	R
6	M7							4			R	R	

* Resistance of each variant to hemagglutination inhibition or neutralization was determined as described previously (Shaw et al., Virology (1986) 155:434-451. Hemagglutination titers were determined for each N-mAb against RRV and the VP3 variant viruses. Variants were considered to be resistant (R) if the HI titer of the N-mAb against the variant was ≥ 8-fold less than the titer of the N-mAb against RRV. Since mAb M2 has little HI activity, data for the M2 mAb represents a neutralization test versus RRV and indicated variants.

† Variant viruses are named for the selecting mAb. GAAD is the quadruple (2G4, 1A9, 7A12, 5D9) variant.

Each mutant gene 4 was sequenced completely using a series of negative strand synthetic oligonucleotides generated during sequencing of the parental RRV gene 4. Each variant gene 4 contained a single base change specifying a single AA change from that of the parental strain (Table 1). The mutations selected by neutralizing mAbs are listed in Table 1 and have been grouped into 6 distinct regions. The resulting amino acid substitutions, which allow variants to escape neutralization, were of 5 separate types. Six of the residue changes involved charge changes in the protein. Substitution of a proline residue in the protein was the next most abundant substitution observed. Other changes either involved the transition of a short side chained residue to an aromatic AA or the conversion of one aromatic AA to another. Only one transition from a long side chained AA to a short side chained residue was observed.

Variant Mutations Correlate With Their Serologic Grouping

The antigenic characteristics of the variants selected in this study are presented in Table 3. Variants were grouped by their resistance to N-mAbs in hemagglutination inhibition (HI) and neutralization assays. The location of base changes in the selected variants was correlated with their serologic grouping. N-mAbs A1, A15 and M11 were closely related by reciprocal HI and neutralization analyses and each selects a mutation in the same region of VP8 (Table 1). Similar findings were observed for variants M14 and 5C4 (region 4) and variants 2G4 and M7 (region 6). Other variants appeared to identify unique regions on the protein and cross-reacted poorly with other serological groups.

Multiple N-mAb Mutant Selection

In the multiple variant, neutralizing mAbs were used sequentially in separate variant selections. The quadruple mutant was expected to be either a composite of the individual mutant changes or a mutant in which one or more of the individual mutations had caused a subsequent N-mAb to select for a new gene 4 mutation. The mutations selected by 2G4 and 1A9 were identified in the same positions as the individual N-mAb variant mutations (Table 1). A mutation at the original 7A12 locus (AA 188) was not found in the quadruple mutant. Instead, a change was identified at AA 173. Similarly, a change at AA 135 was identified in the quadruple mutant but not at the site of the individual 5D9 mutation (114). The intermediate double (2G4/1A9) and triple (2G4/1A9/7A12) selected mutants were also sequenced to determine which N-mAb selected the newly observed mutations. The double mutant contained only the 2G4 and 1A9 changes. With the added selection of N-mAb 7A12, the triple mutant contained a mutation at AA 173. As a result, the new mutation at AA 135 in the quadruple mutant was attributed to the 5D9 selecting N-mAb.

Specificity of the Neutralization Sites

The strain specificity of each N-mAb was determined by neutralization tests with selected viruses of serotypes 1 to 6 (Table 1). Most of the mAbs neutralize only RRV or combinations of RRV and one other strain of rotavirus. The mutations selected by the mAbs with limited strain specificity occur in VP8. The neutralization region identified in VP5 (region 6) was selected by three N-mAbs which neutralize a wide variety of serotypically distinct rotavirus strains (Table 1).

Analysis of Results

Analysis of the RRV sequence and a comparison with other gene 4, VP3 sequences (Lopez et al., Virology (1985) 144:11-19; Lopez et al., Nucleic Acids Res. (1987) 15:4691; and Kantharidis et al., Arch. Virol. (1987) 93:111-121) demonstrated several findings. The RRV gene 4 shared one striking region of nucleic acid conservation with SA11 and RV-5. The first 33 nucleotides of RRV, SA11 and RV-5 gene 4 are identical (except for 2 bases in SA11). In contrast, only 11 bases are conserved at the 3' end of gene 4 of RRV, SA11 and RV-5. There are no long internal regions of nucleic acid conservation. Analysis of sequence information from several other rotavirus genes (Dyall-Smith et al., Nucleic Acids Res. (1984) 12:3973-3982; Ward et al., Virology (1985) 144:328-336; and Dyall-Smith et al., Nucleic Acids Res. (1983) 11:3351-3355) also demonstrates long stretches of conservation at the 5' but not the 3' terminus.

Further conservation of encoded amino acids is found at positions flanking the VP3 trypsin cleavage sites (Table 2). The preferred trypsin cleavage site identified by Lopez et al. occurs after the arginine residue at AA 247 (Lopez et al, Virology (1985) 144:11-19). All rotavirus VP3 proteins studied (Lopez et al., supra; Kantharidis et al., supra; Lopez et al., Virology (1986) 154:224-227) contain this trypsin cleavage site (R + AQ). The regions flanking the trypsin cleavage sites, AA 224 to 236 and 257 to 271, are 100% conserved in RRV, SA11 and RV-5 and could serve to hold these sites in the proper conformation for cleavage.

RRV, RV-5 (Kantharidis et al., supra) and all human strains studied thus far (Gorziglia et al., supra and Lopez et al., supra), lack a proline following the trypsin cleavage site. Five cysteine residues exist in RRV VP3 and all of these are conserved in SA11. The AA

203 residue is the only cysteine that is not conserved in RV-5. In fact, all human rotavirus strains lack a cysteine at AA 203 (Kantharidis et al., supra and Gorziglia et al., supra). Sequence analysis of eleven distinct VP3 variants as well as sequential double, triple, and quadruple mutants has identified six sites in VP3 involved in rotavirus neutralization (Table 1). Strain specific neutralization sites were found in VP8 at AA residues 100, 114-135, and 173-188 while AA 87-89 and 148-150 were associated with limited cross-reactive neutralization. Site 6 (AA 388-393) in VP5 was linked to highly serotype cross-reactive neutralization. mAb 7A12 and 5D9 selected new mutations in the quadruple variant when compared to individual mAb selected mutants (Table 1).

All but one of the mAbs used in this study efficiently inhibit viral hemagglutination as well as neutralize infectivity. Since mAb selected mutations have been identified in several regions on VP3, sites on both VP8 and VP5 were shown to contribute to viral hemagglutination and neutralization. Neutralization escape mutations were spread broadly over the coding region of VP8 although no mutants were identified in or near the trypsin cleavage site. In keeping with the strain specificity of the selecting mAbs, the VP8 mutations tended to occur in areas of considerable sequence diversity (Table 2). However, VP3 directed monoclonals M7, 2G4 and M2 neutralize a variety of serotypically distinct rotavirus strains (Table 1). These monoclonals selected mutations in a conserved area of VP5 (Table 2).

Although group A rotaviruses do not normally form syncytia during viral growth, infection with group B rotavirus, avian reovirus and Nelson Bay virus is associated with membrane fusion (Theil et al., J. Clin. Microbiol. (1985) 21:844-846 and Wilcox et al., Virology (1982) 123:312). Trypsin cleavage of VP3

leads to the rapid enhancement of viral infectivity apparently in association with the passage of virus through the plasma membrane (Kaljot et al., Clin. Res. (1985) 34:677A (abstract) and Suzuki et al., Arch. Virol. (1986) 91:135-144). Trypsin treated virus can also increase plasma membrane permeability (Kaljot et al., supra). The VP3 AA sequence was studied to see if similarities to known fusion proteins could be identified. A long hydrophobic region was not found at the VP8 or VP5 amino termini. However, a region of specific homology with internal fusion protein sites from Semliki Forest virus and Sindbis virus was detected in VP5 (White et al., Quart. Rev. Biophys. (1983) 16:151-195 and Kondor-Koch et al., J. Cell Biol. (1983) 97:644-650) (Table 4).

TABLE 4

Fusion Peptide Homology

					*					*								
RRV	D	Y	S	F	A	L	P	V	G	Q	W	P	V	M	T	G	G	A
	o	o							o			o		o		o	o	o
	o	o	o			o	o		o		o	o	o	o		o	o	o
Sindbis	D	Y	T	C	K	V	F	G	G	V	Y	P	F	M	W	G	G	A
	o	o							o		o	o		o		o	o	o
	o	o	o			o	o		o		o	o	o	o		o	o	o
SA11	D	Y	S	F	A	L	P	V	G	N	Y	P	V	M	T	G	G	A

Homology between the putative fusion region of Sindbis virus (White et al., supra) and the VP3 proteins of RRV and SA11 are presented. Identical amino acids are separated by two circles. Single circles represent conservative amino acid changes (polar, nonpolar, charges or aromatic amino acids changes). Mutation sites identified in viral variants 2G4, M7 and M2 are designated by an asterisk.

The conserved putative fusion sequence of the Sindbis virus E1 protein contains 45% identical plus 27% conserved amino acids with the RRV AA 384-401

region of VP5 (Table 2). SA11 and RV-5 contain 72 and 61% conserved plus identical AA in this region, respectively.

Genetic studies and passive transfer studies have clearly shown that antibody to VP3 can efficiently prevent rotavirus illness (Offit et al., J. Virol. (1986) 58:700-703 and Offit et al., J. Virol. (1986) 60:491-496. Recent studies of young children have clearly demonstrated that the epitope defined by mAb 2G4 (region 6) was immunogenic following local immunization with the RRV candidate vaccine. Therefore, the identification of an antigenically conserved region (region 6) on VP3 that participates in viral neutralization provides a sequence which mimics cross-reactive sequences rather than serospecific regions. The region can thus be efficiently manufactured without any risk of possible infection as with attenuated strains and provides a safe and effective vaccine for a number of strains for rotavirus immunization.

Gene 9

Materials and Methods

Monoclonal Antibodies and Viral Variants

The gene 9 segments of RRV and N-mAb variants resistant to VP7 specific N-mAbs were sequenced and the positions of single base, single amino acid changes were defined. RRV and RRV variants were propagated, isolated and purified as previously escribed (Shaw et al., Virol. (1986) 155:434-451). All but one (N-mAb 57-8) used in this study were previously characterized by competitive mAb binding studies and by their ability to recognize N-mAb selected escape mutants (Shaw et al., supra). The 57-8 mAb was derived from a mouse immunized with a type 4 porcine rotavirus (Benfield et al., VII International Congress of Virology Abstracts

(1987) p. 111). Variants v57-8 were made by growing RRV in the presence of mAb 57-8. Escape mutants were selected and plaque purified as previously described (Shaw et al., supra). A total of five variants to mAb 57-8 were derived from five independent selections performed in separate tissue culture plates.

RNA Sequencing

In order to sequence rotavirus gene segments, the endogenous RNA polymerase activity of the viral cores was used to synthesize plus stranded RNA in vitro (Flores et al., J. Virol. (1982) 43:1032-1037). Sequencing reactions were performed directly on genomic plus stranded RNA using reverse transcriptase (Seikagaku America) by the Sanger method (Sanger et al., Proc. Natl. Acad. Sci. (1977) 74:5463-5467; Zagursky et al., Gene Anal. Tech. (1985) 2:5). Initial sequence was obtained from an oligonucleotide primer complementary to the 3' terminus of the SA11 gene 9 which was provided by Michael Dyall-Smith. Sequence was extended across gene 9 by synthesizing a series of negative strand primers, 18 nucleotides in length, at intervals of 200 to 250 nucleotides on an Applied Biosystems oligonucleotide synthesizer. In order to verify sequence in regions of RNA with a high degree of secondary structure, additional plus stranded primers were used to sequence double stranded RNA templates by the method of Bassel-Duby et al., J. Virol. (1986) 60:64-67.

Electrophoresis

In order to study the size and mobility of VP7 of RRV and v3, an [^{35}S] methionine (1236 Ci/mMole; ICN) labeled lysate of rotavirus infected Ma104 cells was made as previously described (Greenberg et al., J. Virol (1983) 47:267-275). Lysates (0.1 ml) were pre-treated with 10 µl of a 50:50 wt:vol dilution of

Protein A sepharose CL-43 beads (Pharmacia) for 30 minutes. Beads were removed by centrifugation and a 1:200 dilution of guinea pig hyperimmune anti-RRV serum was incubated with the supernatant for 1 hour at 4°C. Protein A sepharose was added and the suspension was incubated an additional 30 minutes at 20°C. The sepharose was pelleted and washed three times with lysis buffer. Immunoprecipitated proteins were released from protein A beads by boiling in 50 µl SDS sample buffer and were separated on 10% polyacrylamide gels (19:1). Prior to SDS treatment, endoglycosidase H was used in order to remove carbohydrate side chains from immunoprecipitated glycoproteins according to the manufacturer's directions (Boehringer Mannheim).

Results

Sequence of RRV Gene 9, VP7

The complete nucleotide sequence of the rhesus rotavirus gene 9 is presented in Table 5.

TABLE 5

SEQUENCE OF RRV GENE 9, VP7

```
                1 GGC TTT AAA AGC GAG AAT TTC CGT TTG GCT AGC GGT
RRV             1
SA11

TAG CTC CTT TTA ATG TAT GGT ATT GAA TAT ACC ACA GTT
                  M   Y   G   I   E   Y   T   T   V

CTA ACC TTT CTG ATA TCG CTC ATT CTA TTG AAT TAT ATT
 L   T   F   L   I   S   L   I   L   L   N   Y   I
                                 I

TTA AAA
 L   K

121 TCT TTG ACT AGA ATG ATG GAC TGT ATT ATT TAC AGA
RRV  25  S   L   T   R   M   M   D  [C]  I   I   Y   R
SA11                    I

TTT CTT TTT ATT GTA GTT ATT TTG TCA CCA TTA CTA AAA
 F   L   F   I   V   V   I   L   S   P   L   L   K
 L                   I                       F   R

GCC CAA AAT TAT GGA ATT AAT CTA CCA ATT ACT GGT TCT
 A   Q   N   Y   G   I   N   L   P   I   T   G   S

ATG GAC
 M   D

241 ACT GCA TAC GCT AAC TCT ACA CAG GAA GAG ACT TTT
RRV  65  T   A   Y   A   N   S   T   Q   E   E   T   F
SA11

CTC ACA TCT ACT TTG [TGT] CTA TAT TAT CCA ACT GAA GCT
 L   T   S   T   L  [C]  L   Y   Y   P   T   E   A

GCA ACA GAA ATA AAT GAT AAT TCG TGG AAG GAT ACA CTC
 A   T   E   I   N   D   N   S   W   K   D   T   L

TCA CAA
 S   Q
```

TABLE 5 (continued)

```
                361 TTA TTC TTG ACT AAA GGA TGG CCA ACT GGA TCA GTT
RRV SA11        105  L   F   L   T   K   G   W   P   T   G   S   V

                TAT TTT AAA GAA TAC ACG GAT ATT GCT TCC TTT TCA GTT
                 Y   F   K   E   Y   T   D   I   A   S   F   S   V
                                         N

                GAT CCA CAA CTA TAT TGT GAT TAT AAC GTG GTA CTT ATG
                 D   P   Q   L   Y  [C]  D   Y   N   V   V   L   M

                AAA TAT
                 K   Y

                481 GAT GCG ACT TTG CAG CTG GAC ATG TCT GAA CTT GCT
RRV SA11        145  D   A   T   L   Q   L   D   M   S   E   L   A

                GAT TTA ATA CTG AAT GAA TGG CTG TGC AAT CCA ATG GAT
                 D   L   I   L   N   E   W   L  [C]  N   P   M   D

                ATT GCT CTA TAT TAT TAT CAA CAA ACA GAC GAA GCT AAC
                 I   A   L   Y   Y   Y   Q   Q   T   D   E   A   N
                     T

                AAA TGG
                 K   W

                601 ATT TCT ATG GGA TCT TCC TGT ACA ATT AAA GTA TGT
RRV SA11        185  I   S   M   G   S   S  [C]  T   I   K   V  [C]

                CCA CTT AAT ACA CAG ACT CTT GGA ATT GGG TGT TTG ACT
                 P   L   N   T   Q   T   L   G   I   G  [C]  L   T

                ACT GAT ACG GCA ACA TTT GAA GAA GTC GCT ACA GCT GAA
                 T   D   T   A   T   F   E   E   V   A   T   A   E
                         A   T

                AAA CTT
                 K   L
```

TABLE 5 (continued)

```
                721 GTG ATT ACT GAC GTT GTC GAT GGC GTG AAT CAT AAA
RRV             225  V   I   T   D   V   V   D   G   V   N   H   K
SA11

                CTT GAT GTT ACA ACT GCT ACT TGC ACT ATC AGA AAC TGC
                 L   D   V   T   T   A   T  [C]  T   I   R   N  [C]

                AAA AAA TTA GGA CCA AGG GAA AAT GTA GCA GTT ATT CAA
                 K   K   L   G   P   R   E   N   V   A   V   I   Q

                GTT GGA
                 V   G

                841 GGT TCT GAT GTT CTC GAC ATA ACG GCT GAT CCA ACC
RRV             265  G   S   D   V   L   D   I   T   A   D   P   T
SA11                             I

                ACA GCA CCA CAA ACT GAA CGA ATG ATG CGC ATT AAT TGG
                 T   A   P   Q   T   E   R   M   M   R   I   N   W

                AAG AAA TGG TGG CAA GTT TTT TAT ACC GTA GTA GAC TAT
                 K   K   W   W   Q   V   F   Y   T   V   V   D   Y

                GTG AAT
                 V   N
                     D

                961 CAA ATA ATT CAA GCA ATG TCC AAA AGA TCA CGA TCA
RRV             305  Q   I   I   Q   A   M   S   K   R   S   R   S
SA11                                 V

                CTT AAC TCT GCT GCA TTC TAT AAT AGA ATA TAG GTA TAG
                 L   N   S   A   A   F   Y   N   R   I
                                         Y       V

                CTT TGG ATA GAA ATG TAT GAT GTG ACC
```

Gene 9 is 1062 bases long and contains a single long open reading frame which encodes a protein of 326 amino acids. The first methionine start site originates at base 49, and two in frame termination codons end the protein at base 1027. The encoded VP7 protein (37 kD) contains four in-frame methionines in the first 63 amino acids and 9 cysteine residues. The amino terminus contains 2 hydrophobic domains. There is one potential N-linked glycosylation site of the type N X T at amino acid 69. The glycosylation site at amino acid 69 is conserved among all but one rotavirus strain (Both et al., Proc. Natl. Acad. Sci. (USA) (1983) 80:3091-3095; Arias et al., J. Virol. (1984) 50:657-661; Dyall-Smith et al., Nucleic Acids Res. (1984) 12:3973-3982; Green et al., Virology (1987) 161:153-159).

The entire RRV VP7 protein is highly conserved when compared to the VP7 amino acid sequences of other rotaviruses. In particular, all eight of the carboxy terminal cysteine residues are conserved in every reported rotavirus strain (Green et al., supra). The amino terminal most cysteine residue (amino acid 32) is only shared by the simian RRV and SA11 rotavirus strains (Table 5). The VP7 proteins of RRV and SA11 strains are 95% identical, containing only 15 amino acid differences (Table 5). The similarity of these simian rotavirus strains is further demonstrated by the fact that all but two of these changes are conservative amino acid substitutions.

Variants Resistant to Neutralizing mAbs

Neutralizing monoclonal antibodies directed at VP7 were used to select antibody resistant RRV variants (Shaw et al., Virol. (1986) 155:434-451). Each mutant gene 9 was sequenced completely using the series of negative strand synthetic oligonucleotides generated during sequencing of the parental RRV gene 9. Each

variant gene 9 contains a single base change which specifies a single amino acid change from the parental strain (Table 6).

TABLE 6

GENE 9 NUCLEOTIDE AND AMINO ACID CHANGES IN VARIANTS SELECTED BY N-MABS

RRV VARIANT	NUCLEOTIDE CHANGE bp #	AMINO ACID CHANGE (AA#)	REGION	SELECTING MAB NEUTRALIZATION SPECIFICITY*
v57-8**	A → G bp 328	Gln → Asp (94)	A	RRV, SA11, NCDV, UK, Gottfried
v4G2	A → G bp 328	Gln → Asp (94)	A	RRV, SA11
v4C3	A → G bp 328	Gln → Asp (94)	A	RRV, SA11
v159	T → G bp 330	Gln → Lys (94)	A	RRV, SA11
v4F8	A → G bp 334	Asn → Asp (96)	A	RRV, SA11
v4F5	T → C bp 337	Ser → Pro (97)	A	RRV, SA11
v3	G → T bp 345	Lys → Asn (99)	A	RRV
v96	G → A bp 679	Asp → Asn (211)	C	RRV
v5H3	G → A bp 679	Asp → Asn (211)	C	RRV
v159/5H3 #	T → G bp 330	Gln → Lys (94)	A	
	A → C bp 682	Thr → Pro (212)	C	

* N-mAbs that selected variants were tested in plaque reduction neutralization tests (focus reduction neutralization for DS1) (Shaw et al., Virology (1986) 155:434-451). Neutralizing mAbs were titered against RRV, SA11, NCDV, OSU, UK DS1, WA, and Gottfried rotavirus strains. Viral neutralization is designated if the titer of the mAb against the indicated viruses is no less than 1/8 of the titer against RRV.

** Five identical v57-8 variants were isolated from five independent mAb 57-8 selections.

v159/5H3 was constructed as a double RRV mutant made sequentially to N-mAbs 159 and 5H3.

The mutations selected by N-mAbs have been grouped into 2 distinct regions, A and C, as previously defined by Dyall-Smith et al., Proc. Natl. Acad. Sci. (USA) (1986) 83:3465-3468. Antibodies selecting mutations in region A were able to compete with or cross-react in reciprocal hemagglutination inhibition or neutralization assays with other region A specific N-mAbs (Shaw et al. (1986), supra). These antibodies selected mutations at amino acids 94 to 99. All variants derived from the five independent mAb 57-8 selections were also found in the A region at base 328, AA 94. The 57-8 mAb efficiently neutralizes viruses of serotypes 3, 4 and 6, while the other mAbs (4G2, 4C3, 159, 4F8, 4F5, 3) are serotype 3- or RRV-specific (Table 6). Competition inhibition studies similar to those previously described (Shaw et al. (1986), supra) demonstrated that mAb 57-8 competed for viral binding with mAbs 159, 3 and 96.

To better localize the mAb 57-8 heterotypic neutralization site on VP7, the amino acid homology of the A and C regions of viral serotypes 1-6 were compared (Table 7).

TABLE 7

A REGION

VIRUS [Serotype]		N-mAb 57-8 Sensitive	90						95									
			T	E	A	A	T	E	I	N	D	N	S	W	K	D	T	
RRV	[3]	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
SA11	[3]	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ST3	[4]	+	S	-	-	P	-	Q	-	S	-	T	E	-	-	-	-	
VA70	[4]	+	S	-	-	P	-	Q	-	S	-	-	E	-	-	-	-	
UK	[6]	+	V	-	-	S	N	-	-	A	-	T	E	-	-	-	-	
NCDV	[6]	+	V	-	-	S	N	-	-	A	-	T	E	-	-	-	-	
Wa	[1]	-	-	-	-	S	-	Q	-		-	G	D	-	-	-	S	
M37	[1]	-	-	-	-	S	-	Q	-	S	-	-	E	-	-	-	S	
Hu5	[2]	-	A	-	-	K	N	-	-	S	-	D	E	-	E	N	-	
OSU	[5]	-	N	-	-	-	-	-	-	A	-	T	K	-	T	E	-	

C REGION

			210					215					220			
			T	T	D	T	A	T	F	E	E	V	A	T	A	
RRV	[3]	+	-	-	-	A	T	-	-	-		-	-	-	-	
SA11	[3]	+	Q	-	N	-	-	-	-	-	T	-	-	D	S	
ST3	[4]	+	Q	-	N	V	-	-	-	-	M	-	-	D	S	
VA70	[4]	+	L	-	N	P	D	-	-	-	T	-	-	-	T	
UK	[6]	+	I	-	N	P	D	-	-	-	T	-	-	-	M	
NCDV	[6]	+														
Wa	[1]	-	-	-	N	V	D	S	-	-	M	I	-	E	N	
M37	[1]	-	-	-	N	V	D	S	-	-	M	-	-	E	N	
Hu5	[2]	-	-	-	-	V	N	-	-	-	I	-	-	S	S	
OSU	[5]	-	-	-	-	I	N	S	-	-	T	-	-	N	-	

A comparison of the amino acid sequences of VP7 regions A and C in 10 rotavirus strains resistant (serotypes 1, 2 and 5) or sensitive serotypes 3, 4 and 6) to neutralizing mAb 57-8 (Benfield et al., supra). Amino acid sequences of the VP7 A and C regions from Wa (Richardson et al., J. Virol. (1984) 51:860-862); Mason et al., Virus Res. (1985) 2:291-299), Hu5 (Dyall-Smith et al. (1984), supra), OSU (Gorziglia et al., J. Gen. Virol. (1986) 67:313-320), UK (Elleman et al., Nucleic Acids Res. (1983) 11:4689-4701), NCDV (Glass et al., Virology (1985) 141:292-298), SA11 (Both et al., Proc. Natl. Acad. Sci. (USA) (1983) 80:3091-3095; Arias et al., J. Virol. (1984) 50:657-661), and M37, ST3 and VA70 (Green et al., Virology (1987) 161:153-159) were compared to the A and C regions of the RRV. N-mAb escape mutants contain single amino acid changes at positions designated by an asterisk. N-mAb 57-8 independently selected 5 region A RRV mutants with single substitutions at amino acid 94. Amino acids conserved among rotaviruses are boxed. Dashes (-) indicate identical amino acids.

Serotype 4 (ST3 and VA70) and serotype 6 (NCDV and UK) viruses were homologous with the serotype 3 RRV and SA11 rotaviruses in only 8 of 15 amino acids in region A (amino acids 87-101). Similarly, the C region of serotype 4 and 6 viruses contained only 6 of 13 identical amino acids (amino acids 209-221) with the C region of serotype 3 viruses. Within the A and C regions, amino acids 88, 89, 93, 95, 98, 210, 215, 216, and 219 are conserved in all rotavirus strains (Green et al., supra). The serotype 3, 4 and 6 viruses also have conserved amino acids at positions 99-101, 214 and

218 while serotype 1, 2 or 5 strains substitute different amino acids at these positions (Table 7) and are not neutralized by mAb 57-8.

To determine the effect of region A, escape mutants on the specificity of region C N-mAbs, double N-mAb resistant variant was selected (Table 6). This variant was first selected in the presence of the 159 N-mAb. Subsequently, the 159 variant was grown in the presence of the 5H3 N-mAb. The resulting double variant was selected and sequenced.

The first mutation was identical to the RRV variant 159 (amino acid 94 Gln to Lys) in the A region. A second mutation was observed at amino acid 212 in the C region. This mutation was one amino acid away from the singly selected variant v5H3 mutation site. The initial A region mutation may have altered the C region specificity of the second selecting N-mAb or the 5H3 N-mAb can select for mutations at several closely associated amino acid locations in the C region.

Neutralizing mAb 3 selected for a mutation in the A region. Variant v3 contained a single mutation in the amino acid 99 codon which changes a lysine to an asparagine residue and creates a potential N-linked glycosylation site (Table 8). When variant v3 and the parental RRV were compared by SDS-PAGE, the v3 variant VP7 protein showed reduced mobility relative to the parental RRV VP7. In particular, Ma-104 cells were infected with RRV or variant v3 at zero time. At three hours post infection, cells were starved for one hour in methionine-free media in the presence of 1 µg/ml of actinomycin D. Infected cells were labeled with 250 µCi/ml [^{35}S]- methionine for 6 hours at which time cell lysates were prepared as previously described (Greenberg et al., J. Virol. (1983) 47:267-275). Hyperimmune RRV antiserum and protein A sepharose beads were used to immunoprecipitate rotavirus proteins

(Greenberg et al., J. Gen. Virol. (1983) 64:313-324) and individual proteins were separated by SDS PAGE (15%) analysis (O'Farrell, J. Biol. Chem. (1975) 250:4007-4021). The location of the glycosylated form of VP7 in the RRV and v3 viruses as shown on an autoradiogram indicated that variant 3 had decreased mobility.

Analysis of Results

The data demonstrates that the neutralizing epitopes on the RRV VP7 are located in the A and C regions described for the SA11 rotavirus (Dyall-Smith et al. (1986), supra). Five serotype 3 specific mAbs selected single base, single amino acid changes in the previously defined A region of VP7 (Table 6). Three RRV specific mAbs (3, 96, 5H3) selected mutations in the A or C regions (Table 2). Mutations in the previously described B region (amino acid 147) were not identified in this study (Dyall-Smith et al. (1986), supra).

Although several studies recently emphasized the importance of glycosylation in the antigenicity of viral proteins, including the SA11 VP7 (Caust et al., Arch. Virol. (1987) 96:123-134), amino acid and glycosylation changes in the VP7 of v3 do not markedly alter the VP7 antigenicity. MAbs 159 and 4G2 directed at the A region as well as mAbs 5H3 and 96 directed at the c region were equally reactive with v3 and the parental RRV (Shaw et al. (1986), supra). Additional glycosylation and an amino acid change at residue 99 did not dramatically affect antibody binding to either the A or C region.

Heterotypic epitopes on VP7 were present even though most VP7 directed monoclonal antibodies were serotype or strain specific (Coulson et al., J. Virol. (1985) 54:14-20; Benfield et al., supra).

The sequence analysis of mAb 57-8 variants demonstrated that homotypic and heterotypic domains on VP7 were closely associated. Since there was not a continuous stretch of amino acids in either the A or C region (Table 7), mAb 57-8 may have recognized conserved parts of both the A and C regions of serotype 3, 4 and 6 viruses.

Expression of RRV Proteins

Construction of VP3 β-galactosidase Expression Vector

Plasmid 4R20 was prepared in the following manner. RRV was purified by CsCl density gradient centrifugation. Viral cores were activated by treatment with EDTA. Plus stranded RNA was synthesized using the endogenous viral RNA-dependent RNA polymerase. Specific gene 4 primers were annealed to the RNA and gene 4 was reverse transcribed to produce cDNA using avian myeloblastosis virus reverse transcriptase (Seikegaku America). The second cDNA strand was synthesized using a combination of DNA polymerase I (E. coli) and RNase H. The ds cDNA was ligated to BamHI linkers and subsequently digested with BamHI and KpnI. (Gene 4 has a unique KpnI site at base 1433). In this way, the two ends of gene 4 were cloned into the BamHI and KpnI cut vector pUC19 (BRL). Clones were screened by hybridization. The plasmid 4R20 was determined to contain the 5' end of gene 4.

A β-galactosidase expression vector was prepared in a pUC18 plasmid vector (Bethesda Research Labs) by digestion of the plasmid p4R20 which contains bases 500 to 1433 of gene 4, with KpnI and EcoRI. The resultant 383 base internal fragment of gene 4 was directionally ligated into pUC18. The result was a

gene encoding a β-galactosidase-VP3 fusion protein with the in-frame insertion of the sequence encoding VP3 at both the EcoRI and KpnI sites.

```
ATG ACC ATG ATT ACG     GTA CCC .......β-GALACTOSIDASE GENE
  T   M   I   T         V   P  .......  β-GAL PROTEIN

AAT TCT....TCA TTG      RRV GENE 4, BASE 1051-1433
  N   S .... S   L      RRV VP3, AA 348-473
```

Construction of VP3/Salmonella flagellin Expression Vector

The Salmonella flagellin gene in plasmid p402 and p404 was provided by Dr. Bruce Stocker of Stanford University. In order to insert portions of VP3 in-frame into the flagellin gene, p402 and p404 were digested with the EcoRV restriction enzyme and an XhoI oligonucleotide linker having the sequence CCTCGAGG was ligated at that site. The resultant p402XI and p404XI were linearized by digestion with XhoI and two complementary oligonucleotide linkers were inserted. The oligonucleotide sequences are as follows:

```
5'TCGAGGGGGTGATTATAGCTTTGCATTACCGGTTGGTCAATACCCAGTAATGACTGGCGGAGCA3'
3'    CCCCCACTAATATCGAAACGTAATGGCCAACCAGTTATGGGTCATTACTGACCGCCTCGTAGCT5'
```

Oligonucleotide linkers, 64 bases in length, were synthesized chemically on an Applied Biosystems oligonucleotide synthesizer and gel purified. The combination of these complementary oligonucleotides resulted in XhoI sticky ends on a fragment containing the entire putative fusion region of VP3 (bases 1153-1212, amino acids 382-401).

Construction of VP7 Expression Vector

The RRV gene 9 sequence encoding VP7 was cloned into pUC19 and pT7T3 18U (available commercially from Stratagene). Cloning was performed as for VP3, gene 4, except that BamHI linkered cDNA was inserted directly into pUC19 or pT8T3 18U that were opened at their BamHI sites.

Expression of VP3 Fusion Proteins

The expression construct encoding the VP3/β-galactosidase fusion protein was transformed into DH5-α cells (BRL). The sequence of each construct was determined using oligodirected DNA sequencing by the Sanger method. Each construct is in reading frame with the β-galactosidase protein and disrupts β-galactosidase expression as demonstrated by SDS-PAGE.

The expression construct encoding the VP3/flagellin fusion protein was transformed into DH5-α cells. The in reading frame insertion of the gene 4 oligonucleotide primer pair was determined by direct DNA sequencing (Sanger) using a primer made to the Salmonella flagellin gene. The expression of the fused VP3/flagellin product is currently being studied in E. coli and in Salmonella.

Localization of the conserved regions of RRV, particularly the serotype-specific and cross-reactive neutralizing domains, provided for preparation of polypeptide compositions which are useful as vaccines. The polypeptide vaccines are advantageous over use of attenuated virus vaccines as the peptide compositions do not contain infectious material.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to

the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A polypeptide composition comprising a substantially pure preparation of rhesus rotavirus (RRV) VP3 or VP7 or immunologically active fragments thereof.

2. A polypeptide composition of Claim 1 comprising at least one fragment of rhesus rotavirus VP3 or VP7 wherein said fragment comprises a sequence homologous to at least about 90% of the sequence of said RRV protein.

3. A polypeptide composition of Claim 1 comprising at least one fragment of rhesus rotavirus VP3 or VP7 wherein said fragment comprises not more than about 100 amino acids.

4. A polypeptide composition of Claim 1 wherein said composition comprises at least one polypeptide having the same amino acid sequence as at least about 10 consecutive amino acids within residues 75-200 or 370-490 of VP3 or residues 75-250 of VP7 of rhesus rotavirus and sequences immunologically cross-reactive therewith.

5. A polypeptide composition of Claim 4 wherein said polypeptide has the same amino acid sequence as at least about 15 consecutive amino acids located within residues 75-200, 380-416 or 451-480 of VP3 or 75-125 or 200-230 of VP7 of rhesus rotavirus and sequences immunologically cross-reactive therewith, wherein said immunologically cross-reactive sequences differ from the wild type sequence by not more than three lesions.

6. A polypeptide composition of Claim 5 wherein said polypeptide comprises residues 82-94, 95-105, 109-119, 143-155, 183-193, 383-393 or 388-398 of VP3 or 89-99, 91-102, 94-104 or 206-217 of VP7.

7. A polypeptide composition of Claim 5 wherein said polypeptide comprises residues 77-99, 90-110, 104-124, 125-145, 138-160, 163-183, 378-398 or 383-403 of VP3 or 84-104, 86-107, 89-109 or 201-222 of VP7.

8. A polypeptide composition of Claim 3 having at least one polypeptide which is chemically synthesized and comprises not more than about 50 amino acids.

9. A polypeptide composition of Claim 5 wherein said rhesus rotavirus sequence is joined to a heterologous protein or fragment thereof comprising at least about 15 amino acids.

10. A polypeptide composition of Claim 9 wherein said heterologous protein provides for enhanced immunogenicity.

11. A polypeptide composition of Claim 10 wherein said polypeptide is produced recombinantly.

12. A polypeptide composition of Claim 11 wherein said heterologous protein comprises all or a portion of Salmonella flagellin or β-galactosidase.

13. A polypeptide composition comprising a substantially pure preparation of rhesus rotavirus VP3 or VP7 or fragments thereof comprising the same amino acid sequence as at least about 15 consecutive amino acids within residues 75-200 or 380-416 comprising

residues 82-94, 95-105, 109-119, 143-155, 183-193, 383-393 or 388-398 of VP3 or 84-104, 86-107, 89-109 or 201-222 of VP7 and sequences cross-reactive therewith comprising not more than three lesions.

14. A polypeptide composition of Claim 13 wherein said polypeptide comprises an amino acid sequence having identical amino acids at residues 87-89, 100, 114, 135, 148, 150, 173, 188, 388 and 393 of VP3 and 94, 96, 97, 99, 211 and 212 of VP7.

15. A polypeptide of from about 15 to about 35 amino acids comprising the same amino acid sequence as at least about 15 consecutive amino acids located within residues of VP3 of rhesus rotavirus designated:

(a) 380-416 or 451-480 of VP3 of rhesus rotavirus;

(b) 75-200 of VP3 of rhesus rotavirus and comprising residues 82-94, 95-105, 109-119, 143-155 or 183-193; or

(c) 75-125 or 200-230 of VP7 of rhesus rotavirus and comprising residues 94, 96, 97, 99, 211 or 212;

and sequences immunologically cross-reactive therewith, wherein said immunologically cross-reactive sequences differ from the wild type sequence by not more than three lesions.

16. A polypeptide of Claim 15 wherein any of said lesions comprise conservative substitutions.

17. A polypeptide of Claim 15 wherein said polypeptide comprises identical amino acids at residues 87-89, 100, 114, 135, 148, 150, 173, 188, 388 and 393.

18. An expression cassette comprising a DNA sequence encoding VP3 or VP7 of rhesus rotavirus under the transcriptional control of a promoter which is

functional in a host cell of interest, wherein said promoter is joined to other than native DNA.

19. An expression casette of Claim 18 wherein said promoter is functional in prokaryotic cells.

20. An expression cassette of Claim 19 additionally comprising in reading frame with said DNA sequence encoding VP3 or VP7 a DNA sequence encoding a heterologous protein or fragment thereof having at least about 15 amino acids.

21. An expression cassette of Claim 20 wherein said heterologous protein comprises β-galactosidase or Salmonella flagellin.

22. A vaccine comprising in a physiologically suitable diluent a polypeptide composition comprising a substantially pure preparation of rhesus rotavirus VP3 or VP7 or immunologically active fragments thereof in an amount effective to induce antibodies.

23. A vaccine of Claim 22 wherein said composition comprises a polypeptide comprising at least 10 amino acids comprising the same amino acid sequence as consecutive amino acids within residues 75-200 or 370-490 of VP3 or residues 75-250 of VP7 of rhesus rotavirus and sequences immunologically cross-reactive therewith.

24. A vaccine comprising a polypeptide of fewer than about 50 amino acids comprising the same amino acid sequence as at least about 15 consecutive amino acids within residues 75-200 or 380-416 of VP3 or 75-125 or 200-230 of VP7 comprising residues 82-94, 95-105, 109-119, 143-155, 183-193, 383-393 or 388-398 of VP3 or 89-99, 91-102, 94-104 or 206-217 of VP7 and

sequences immunologically cross-reactive therewith, wherein said immunologically cross-reactive sequences differ from the wild type sequence by not more than three lesions.

25. A vaccine of Claim 24 wherein said polypeptide comprises identical amino acids at residues 87-89, 100, 114, 135, 148, 150, 173, 188, 388 and 393 of VP3 and 94, 96, 97, 99, 211 and 212 of VP7.

26. A method of protecting a susceptible host from rotavirus infection comprising adminstering to said host a vaccine comprising in a physiologically suitable diluent in an amount effective to induce antibodies, a polypeptide composition comprising a substantially pure preparation of rhesus rotavirus VP3 or VP7 or immunologically active fragments thereof.

27. A method of protecting a susceptible host from rotavirus infection comprising adminstering to said host a vaccine comprising a polypeptide of about 15 to about 35 amino acids comprising the same amino acid sequence as at least about 15 consecutive amino acids located within residues of VP3 of rhesus rotavirus designated:

(a) 380-416 or 451-480 of VP3 of rhesus rotavirus;

(b) 75-200 of VP3 of rhesus rotavirus and comprising residues 82-94, 95-105, 109-119, 143-155 or 183-193; or

(c) 75-125 or 200-230 of VP7 of rhesus rotavirus and comprising residues 94, 96, 97, 99, 211 or 212;

and sequences immunologically cross-reactive therewith, wherein said immunologically cross-reactive sequences differ from the wild type sequence by not more than three lesions.

INTERNATIONAL SEARCH REPO

International Application No PCT/US89/00018

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 39/12, 37/02;C07K 7/06,08,10; C12N 7/00

U.S. CL.: 424/89; 514/14,15; 530/326,327,328,350; 435/320;536/27

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	424/89; 514/14,15; 530/326,327,328,350; 435/320; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
A	US, A, 4,571,385, 18 February 1986, (GREENBERG), See the entire document.	1-27
A	Journal of Virology, Vol. 60, No. 2, issued November 1986, P. OFFIT, "Reassortant Rotaviruses containing structural Proteins VP and VP7 from different parents induce antibodies Protective against each parental serotype", pp. 491-496, see the entire document.	1-27
Y	Journal of Virology, Vol. 39, No. 3 issued September 1981, M. ESTES, "Proteolytic Enhancement of Rotavirus Infectivity: Molecular Mechanisms", pp. 879-888, See the entire document.	1-4
Y	Chemical Abstracts, Volume 105, No. 3, issued 1986 (Columbus, Ohio, USA) D.S.M. Leigh, "Rotavirus", see page 225, column 1, the abstract NO. 19846p, (Univeristy of Melbourne), ~~AU Appl. 24/4,733, 27 April 1984, 25 pp.~~	1-7,9-16, 18-27

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 8
17 April 1989	07 JUN 1989
International Searching Authority 1	**Signature of Authorized Officer 10**
ISA/US	T.D. Wessendorf T.D. WESSENDORF

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Chemical Abstracts, Volume 106, No. 21, issued 1987 (Columbus, Ohio, USA) A. Carlos, "Synthesis in Escherichia coli and immunological characterization of a polypeptide containing the cleavage sites associated with trypsin enhancement of rotavirus SA11 infectivity," see page 545, col. 1, abstract no. 174252f, J. Gen. Virol. 1987, 68(3), 633-42 (Eng).	1-3,4, 9-11, 18-19
Y	Chemical Abstracts, Volume 108, No. 23, issued 1988 (Columbus, Ohio, USA) T.M. Barnes, "Preparation by chemical or recombinant DNA techniques of immunogen-carrier molecule conjugates which potentiate immune response to the immunogen", see page 186, col. 1, abstract no. 199492p., (Bioenterprises Pty. Ltd), Au Appl. 86/5,559, 21 April 1986, 40 pages.	1-4, 9-11, 18-20
Y	Chemical Abstracts, Volume 109, No. 5, issued 1988 (Columbus, Ohio, USA), R.E. Smith, "Cloning Sequencing, and expression of rotavirus SA-11 major outer capsid protein VP7 cDNA in a baculovirus-insect cell expression system", see page 183, col. 2 abstract no. 33223x, (Abbott Laboratories), Eur. Pat. Appl. EP 251,467, 07 January 1988.	1-6,11, 13,14,18, 19,22,23, 26
P,X	Chemical Abstracts, Volume 109, No. 5, issued 1988 (Columbus, Ohio, USA), E.R. Mackow, "The rhesus rotavirus gene encoding protein VP3: location of amino acids involved in homologous and heterologous rotavirus neutralization and identification of a putative fusion region", see page 150, col. 1, abstractno. 32880x, Proc. Natl. Acad. Sci. U.S.A. 1988, 85(3),645-9 (Eng).	1-20
Y,E	Chemical Abstracts, Volume 110, No. 1, issued 1989, (Columbus, Ohio USA), H.J. Streckert, "A synthetic peptide corresponding to the cleavage region of VP3 from rotavirus SA 11 induces neutralizing antibodies", see pge 554, col. 2, abstract no. 5842a, J. Virol. 1988, 62(11), 4265-9 (Eng).	1-27

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. X OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I- Claims 1-17 and 22-27 drawn to polypeptides, vaccine and method of protection.

Group II- Claims 18-21, drawn to expression casette.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.